Mutations in a Δ^9–Stearoyl-ACP-Desaturase Gene Are Associated with Enhanced Stearic Acid Levels in Soybean Seeds

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ABSTRACT

Stearic acid (18:0) is typically a minor component of soybean [*Glycine max* (L.) Merr.] oil, accounting for only 2 to 4% of the total fatty acid content. Increasing stearic acid levels of soybean oil would lead to enhanced oxidative stability, potentially reducing the need for hydrogenation, a process leading to the formation of undesirable *trans* fatty acids. Although mutagenesis strategies have been successful in developing soybean germplasm with elevated 18:0 levels in the seed oil, the specific gene mutations responsible for this phenotype were not known. We report a newly identified soybean gene, designated *SACPD-C*, that encodes a unique isoform of Δ^9–stearoyl-ACP-desaturase, the enzyme responsible for converting stearic acid to oleic acid (18:1). High levels of *SACPD-C* transcript were only detected in developing seed tissue, suggesting that the encoded desaturase functions to enhance oleic acid biosynthetic capacity as the immature seed is actively engaged in triacylglycerol production and storage. The participation of *SACPD-C* in storage triacylglycerol synthesis is further supported by the observation of mutations in this gene in two independent sources of elevated 18:0 soybean germplasm, A6 (30% 18:0) and FAM94-41 (9% 18:0). A molecular marker diagnostic for the FAM94-41 *SACPD-C* gene mutation strictly associates with the elevated 18:0 phenotype in a segregating population, and could thus serve as a useful tool in the development of cultivars with oils possessing enhanced oxidative stability.

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Abbreviations: ACP, acyl carrier protein; CAPS, cleaved amplified polymorphic sequence; DAF, days after flowering; EST, expressed sequence tag; PCR, polymerase chain reaction; Δ^9–S-ACP-Des, Δ^9–stearoyl-ACP-desaturase.

For decades, the food industry has relied on hydrogenation to increase the oxidative stability of soybean oil. Because this process produces *trans* fatty acids that have been associated with enhanced risk of coronary heart disease (Hu et al., 1997), developing an oxidatively stable, nonhydrogenated oil is highly desired. The production of solid-fat products, such as margarines, from soybean oil is particularly problematic because extensive hydrogenation is required for these applications. One avenue for achieving the goal of *trans*-free solid-fat products is to utilize soybean germplasm that possesses enhanced saturated fatty acid content. Palmitic acid (16:0), the major saturated fatty acid of commercial soybean oil, has desirable oxidative properties and a high melting point, yet is also associated with negative health consequences as a result of its cholesterogenic nature and its ability to promote arterial thrombosis formation (Hornstra and Kester, 1997; Hu et al., 1997). These negative effects, however, are generally not observed with stearic acid (Kris-Etherton and Yu, 1997; Thijssen et al., 2005). In addition to enhancing oxidative stability, a high-stearate-containing soybean oil may also be welcomed by the confectionery industry because it could

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be used as an economical cocoa butter equivalent (Chang et al., 1990).

The elevated stearic acid seed trait in soybean is controlled by homozygous recessive genetic loci designated either *fas* or *st* (Pantalone et al., 2004). Five elevated stearate lines were developed using chemical or X-ray mutagenesis. Soybean germplasm A81-606085 (*fas*), A6 (fas^a), and FA41545 (fas^b) display seed oil 18:0 phenotypes of 19, 30, and 15%, respectively, in contrast to normal soybeans, which contain 2 to 4% stearic acid. Genetic studies demonstrated that these three *fas* loci are allelic and therefore likely represent independent mutations within the same gene (Graef et al., 1985). Germplasm KK-2 (st_1) and M25 (st_2) possess nonallelic loci that when combined (st_1 st_1 st_2 st_2) elevate 18:0 levels to >35% of the fatty acid content of the seed oil (Rahman et al., 1997). It is not known whether st_1 or st_2 are allelic to the *fas* loci. In addition to the above mentioned soybean lines displaying elevated stearic acid phenotypes derived via mutagenesis, a naturally occurring source has been identified. Germplasm line FAM94-41 carries a natural mutation at a locus designated fas_{nc} that results in a 9% 18:0 phenotype in the seed (Pantalone et al., 2002). Despite the considerable differences in the 18:0 accumulation profiles between FAM94-41 and A6 (9 vs. 30%), genetic analysis demonstrated that fas_{nc} and fas^a are allelic (Pantalone et al., 2002).

Although no experimental evidence has been presented that describes the molecular basis of the elevated stearic acid trait in any soybean line, studies using other plant systems have shown the Δ^9–stearoyl-acyl carrier protein desaturase (Δ^9–S-ACP-Des) enzyme to be pivotal in determining stearic acid accumulation (Knutzon et al., 1992; Zaborowska et al., 2002). Δ^9–S-ACP-Des in plants is a plastid-localized soluble desaturase that catalyzes the conversion of stearic acid into the monounsaturated oleic acid (18:1), which in turn can serve as a precursor in the synthesis of polyunsaturated fatty acids (18:2 and 18:3) (Shanklin and Cahoon, 1998). Therefore, this enzyme plays a key role in the processes that determine the physical properties of the majority of cellular glycerolipids, those utilized as membrane constituents as well as the storage triacylglycerols.

Δ^9–stearoyl-acyl carrier protein desaturase genes have been cloned and characterized from a variety of plant species (Shanklin and Somerville, 1991; Thompson et al., 1991; Cahoon et al., 1998; Whittle et al., 2005; Tong et al., 2006; Kachroo et al., 2007). Recently two soybean Δ^9–S-ACP-Des genes, designated *SACPD-A* and *SACPD-B*, have also been reported (Byfield et al., 2006; Byfield and Upchurch, 2007). Both Δ^9–S-ACP-Des isoforms were detected in all soybean lines surveyed, including the high stearic acid line A6, but no correspondence was reported between these genes and the elevated stearic acid trait. Here we present a previously unidentified soybean Δ^9–S-ACP-Des gene, designated *SACPD-C*, that is specifically expressed during seed development. Defects in the *SACPD-C* gene were observed in the elevated 18:0 soybean lines A6 and FAM94-41. These results define *SACPD-C* as a key target in the development of soybean cultivars containing an elevated stearic acid oil trait.

MATERIALS AND METHODS

Plant Materials

Soybean lines 'Dare', A6, FA8077, and FAM94-41 were used in this study. Dare (*FasFas*) is a normal cultivar that accumulates typical levels of stearic acid in the mature seed (3–4%). The elevated stearate germplasm A6 (fas^afas^a) was generated by sodium azide treatment of breeding line FA8077 and contains greater than sixfold higher levels of 18:0 than normal lines (Hammond and Fehr, 1983). FAM94-41($fas_{ne}fas_{nc}$) possesses a natural mutation that confers two- to threefold higher levels of stearic acid than normal lines (Pantalone et al., 2002). For the fas_{nc} segregation analysis, F_2 seeds from a cross between FAM94-41 and the mid-oleic acid breeding line N98-4445A (Burton et al., 2006) were used.

Gene Isolation and Analysis

Total cellular RNA was isolated from frozen developing seeds of soybean cultivar Dare, using the Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). First-strand cDNA was generated from total RNA using the SuperScript First-Strand Synthesis system for reverse transcription-polymerase chain reaction (Invitrogen). Genomic DNA was extracted using young leaf tissue as previously described (Dewey et al., 1994). To clone both the full-length *SACPD-C* cDNA and its genomic version, primers 5′-ATCTCCAACCTCTCCACAGTTC-3′ and 5′-AGTCACAAAGCCAAAAACCTG-3′, based on the tentative contig TC205834 (www.tigr.org), were utilized in amplification reactions using the Expand Hi-fidelity polymerase chain reaction (PCR) system (Roche Applied Science, Indianapolis, IN). Each 50-μL reaction contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 25 pmol of each primer, 1 U *Taq* DNA polymerase, and 1 to 2 μL of the final first-strand product reaction for cDNA templates, and 50 to 100 ng when genomic DNA was used as template. Each reaction was conducted using the following thermocycling parameters: 94°C incubation for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 1 min (for cDNA; 2 min for genomic DNA), and a final 72°C extension for 7 min. The PCR products were cloned into the pCR 2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen). DNA sequence was obtained using the Iowa State University DNA Facility (www.dna.iastate.edu). Sequence alignments were conducted using bioinformatics programs available in the Bioinformatics Toolkit (2006). Analysis of the translated open reading frame with the ChloroP program (Center for Biological Sequence Analysis, 2007) led to the prediction of an N-terminal chloroplast transit peptide as shown in Fig. 1A. During the course of this study, it became apparent that the GenBank entries for

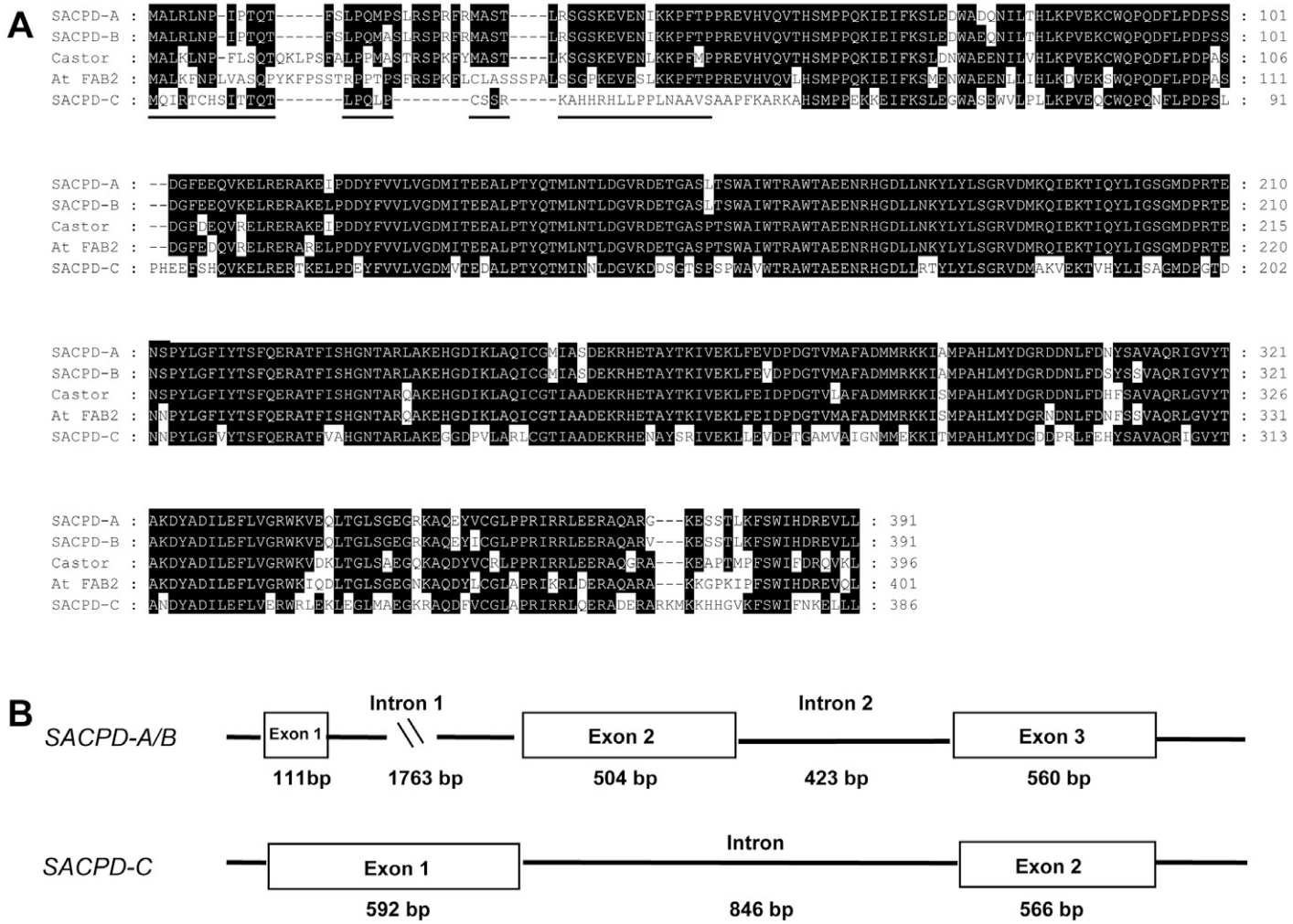

Figure 1. Gene structure and predicted protein sequence of the *SACPD-C* gene of soybean. (A) Alignment of the predicted SACPD-C protein (GenBank no. EF113911) with Δ^9–stearoyl-acyl carrier protein desaturase proteins from soybean (SACPD-A and -B, GenBank entries AY885234 and AY885233, respectively), castor (GenBank no. M59857), and *Arabidopsis* (GenBank no. NP_850400). Residues identical among at least three of the five entries are box shaded. The putative SACPD-C transit peptide that was predicted using the program ChloroP (Center for Biological Sequence Analysis, 2007) is underlined. (B) Comparison of *SACPD-C* gene structure with the *SACPD-A* and *-B* isoforms.

the soybean *SACPD-A* and *SACPD-B* cDNA and predicted protein sequences (AY885234 and AY885233, respectively) contained some errors. These entries have been edited and corrected accordingly.

DNA and RNA Blot Analysis

Ten micrograms of genomic DNA from soybean genotypes Dare, A6, and FA8077 were digested with restriction endonuclease *Hind* III (Promega, Madison, WI) for 3 h. Digested DNA was separated on a 0.8% agarose gel and blotted to a nylon membrane as described (Sambrook and Russell, 2001). A P^{32}–labeled *SACPD-C* cDNA probe was hybridized to the DNA gel blot and washed according to standard protocols (Sambrook and Russell, 2001). Radioactive hybridization signals were detected by exposing the blot to BioMax X-ray film (Eastman Kodak, Rochester, NY). Total cellular RNA was isolated from leaf, flower, root, stem, and developing seed tissue 35 d after flowering (DAF), using the Trizol reagent as outlined by the manufacturer (Invitrogen). RNA blots were generated by separating 10 μg of total cellular RNA on 1.5% agarose-formaldehyde denaturing gels (Sambrook and Russell, 2001). Blotting, hybridization, and wash conditions were the same as described for DNA gel blots.

Molecular Marker Analysis

SACPD-C genomic DNAs were isolated from the progeny of an F_2 population derived from a FAM94-41 × N98-4445A cross using the FastDNA Kit (MP Biomedicals, Solon, OH). Primers 5′-CCACAAAACTTCCTCCCTGA-3′ (forward) and 5′-GAAATGCATACTCACCCAATCA-3′ (reverse) were designed to amplify a 432-bp fragment that encompasses the single nucleotide polymorphism found in the *SACPD-C* gene from FAM94-41 that alters codon 126. PCR components and conditions were the same as described above for cDNA amplifications, except that 60°C was used as the annealing temperature. Eight microliters of amplified product was digested for 2 h at 37°C with the restriction endonuclease *Hga* I (New England Biolabs, Beverly, MA) in 20-μL reaction volumes containing 4 U of enzyme and the recommended buffer. Digested DNA fragments were separated on 1.8% agarose gels stained with

ethidium bromide and visualized using a Gel Logic 100 Imaging System (Eastman Kodak).

Activity Analysis of Recombinant SACPD Enzymes

To obtain mature SACPD-C enzyme suitable for expression in *Escherichia coli* (without the predicted transit peptide), the following sense and antisense primers were used in PCR reactions with cloned full-length cDNA as template: 5′-TTTCATATG-GCGGCGCCGTTC-3′ and 5′-TTTTGTCGACCCTGTT-GACTGACGTTG-3′. Primers were designed to include NdeI and SalI restriction sites to facilitate cloning of the cleaved amplification product into the comparable restriction sites of the pET-24b expression vector (Novagen, San Diego, CA). Plasmids were transformed into host BL21 DE3 and expression was induced as directed by the manufacturer (Novagen). Cells were harvested by centrifugation and resuspended in 2.5 mL of extraction buffer (7 mM Hepes, 7 mM Mes, 7 mM sodium acetate, 6 U mL^{-1} of DNase I, pH 7.4) per gram of cells. Cells were lysed by sonication and clarified by centrifugation at 48,000 *g* for 30 min at 4°C. The supernatant was passaged onto a 1.8-mL column of CM-sephadex, C-50 (Pharmacia, Piscataway, NJ) that had been preequilibrated with 7 mM Hepes, 7 mM Mes, 7 mM sodium acetate, pH 7.4. The column was subsequently washed with 16 mL of equilibration buffer followed by elution with a 60- to 600-mM linear gradient of NaCl (in equilibration buffer). Fractions with the highest concentrations of recombinant protein were concentrated using Centricon YM-30 columns according to the manufacturer's protocol (Millipore Corp., Bedford, MA). Purity of protein was estimated to be >90% by sodium dodecylsulfate polyacrylamide gel electrophoresis. Desaturase preparations were assayed with [1–^{14}C]18:0-ACP substrate with the use of recombinant spinach (*Spinacia oleracea* L.) ACP-I and *Anabaena* vegetative ferredoxin as described in a previous report (Cahoon et al., 1997). Methyl esters of fatty acids were analyzed by argentation thin-layer chromatography and radioactivity in products quantified; assays were performed with three or more replicates.

RESULTS

Identification of a New Soybean Δ^9–Stearoyl-ACP-Desaturase Gene

In an attempt to characterize the number of independent Δ^9–S-ACP-Des genes that are actively expressed in soybean, we conducted TBLASTN alignments of the soybean expressed sequence tag (EST) deposited in GenBank using known plant Δ^9–S-ACP-Des protein sequences as the query. The results of this analysis enabled us to define three discrete classes of Δ^9–S-ACP-Des–like ESTs (data not shown). Two classes corresponded to the recently characterized, and closely related, *SACPD-A* and *SACPD-B* genes (Byfield et al., 2006). The third class formed a unique group that is represented in The Institute for Genomic Research Soybean Gene Index as tentative contig TC205834 (www.tigr.org). Interestingly, 25 of the 27 ESTs that compose TC205834 originated from libraries generated from developing seed tissue or somatic embryos (cultured cells that mimic soybean seed development). In contrast, the ESTs corresponding to *SACPD-A* or *SACPD-B* were obtained from cDNA libraries representing a diversity of plant tissues and/or stages of development (data not shown).

To obtain a full-length cDNA clone of this new Δ^9–S-ACP-Des gene, which we designated *SACPD-C*, PCR amplifications were conducting using primers based on the putative 5′- and 3′- untranslated regions of TC205834. As shown in Fig. 1, *SACPD-C* is predicted to encode a 386-amino-acid-long protein that shares approximately 63% sequence identity with both of the 391-amino-acid-long SACPD-A and SACPD-B proteins. Similar to other plant Δ^9–S-ACP-Des enzymes, SACPD-C possesses a predicted N-terminal transit peptide to facilitate transport into the plastid (Fig. 1A). The SACPD-C protein sequence also contains the structural motifs typical of soluble acyl-ACP-desaturases, including a di-iron center buried within two pairs of antiparallel helices (data not shown).

The same PCR primers used to amplify the full-length *SACPD-C* cDNA were also successful in recovering the corresponding genomic sequence of the gene. *SACPD-C* is composed of two exons separated by an 846-bp intron (Fig. 1B). Structurally, *SACPD-C* differs from the *SACPD-A* and *SACPD-B* genes in that it lacks the large intron located immediately after the putative transit peptide-encoding region (Fig. 1B). The sole *SACPD-C* intron is located at the same position as *SACPD-A/B* intron 2 but is twice the size.

Spatial Distribution of *SACPD-C* Transcripts

RNA blot analysis was conducted to determine the relative abundance of *SACPD-C* transcripts in developing seeds (35 DAF), young leaves, flowers, roots, and stems from soybean cultivar Dare. As shown in Fig. 2, a strong hybridization signal was observed with RNAs isolated from developing seeds. In contrast, negligible signal was observed using RNA preparations from the other tissues tested. These results suggest that *SACPD-C* is under the regulatory control of a highly seed-specific promoter. The RNA blotting results are also consistent with the above mentioned observation that the preponderance of the *SACPD-C* ESTs found in GenBank were derived from developing seed (and somatic embryo) cDNA libraries.

SACPD-C Is Deleted in Mutant Line A6

Given the seed specificity of its expression profile, we speculated that *SACPD-C* functions to enhance the 18:0-ACP desaturation capacity of the immature seed to accommodate the great increase in fatty acid biosynthesis that occurs with the production of storage triacylglycerol reserves. As such, *SACPD-C* would represent a viable candidate gene for mutant soybean germplasm displaying an elevated 18:0

phenotype. The *fas*[a] locus found in the mutant line A6 confers the highest 18:0 seed phenotype (30% total fatty acid content) of any single soybean locus characterized to date. To determine whether mutations in the *SACPD-C* gene may be responsible for this phenotype, we initially attempted to amplify *SACPD-C* from A6 using developing seed cDNA as template. Using the same primers and conditions that were successful in recovering the *SACPD-C* cDNA from cultivar Dare, we were unable to detect the expected product from A6 (Fig. 3A). Occasionally, a faint band of much smaller size is observed when amplifying A6 cDNA, the nature of which is unknown. Consistent with the PCR results, RNA blot analysis showed no detectable hybridization to 35-DAF developing seed RNA isolated from A6 when radiolabeled *SACPD-C* was used as a probe (Fig. 3B). To confirm that the absence of *SACPD-C* gene expression was truly the result of mutagenesis treatment, as opposed to natural cultivar variation, RNAs from breeding line FA8077, the parental line that was mutated to produce A6 (Hammond and Fehr, 1983), were included in this study. As shown in Fig. 3B, normal levels of *SACPD-C* transcripts are observed in genotype FA8077.

To test whether our failure to detect evidence of *SACPD-C* gene expression in A6 could be explained by a gross change in *SACPD-C* gene structure within the A6 genome (such as a deletion, insertion, or rearrangement), Southern blotting experiments were conducted using genomic DNAs isolated from Dare, A6, and FA8077. Regardless of the restriction enzyme used in these analyses, the most intensely hybridizing band (or bands) was always missing from genomic DNA preparations of A6 in comparison with Dare or FA8077. A typical example is shown in Fig. 3C, using the restriction enzyme *Hind* III. Although additional cross-hybridizing bands were shared among all three genotypes, they appeared not to be contributing measurably to the transcript pool in A6 (Fig. 3B). Cumulatively, the RNA and DNA gel blotting results present compelling evidence that the *SACPD-C* gene has been deleted in the A6 mutant germplasm.

A Radical Amino Acid Substitution Is Found in the *SACPD-C* Gene of FAM94-41

Although the 18:0 phenotypes of the A6 and FAM94-41 germplasm lines differ substantially (30 vs. 9% 18:0), allelism studies led to the prediction that the elevated stearate content in both lines were due to independent mutations within the same gene (Pantalone et al., 2002). Because we observed no difficulties in amplifying the expected *SACPD-C* product from FAM94-41, and transcript accumulation levels appeared normal (Fig. 3A and B), DNA sequence analysis was conducted to determine whether more subtle differences were apparent in the FAM94-41 *SACPD-C* gene. Sequence comparisons revealed two polymorphisms between the *SACPD-C* genes from normal cultivar Dare vs. FAM94-41. One polymorphism, a G→A substitution at position 1079 (with respect to the *SACPD-C* start codon) does not alter the predicted amino acid sequence. The other polymorphism, however, results

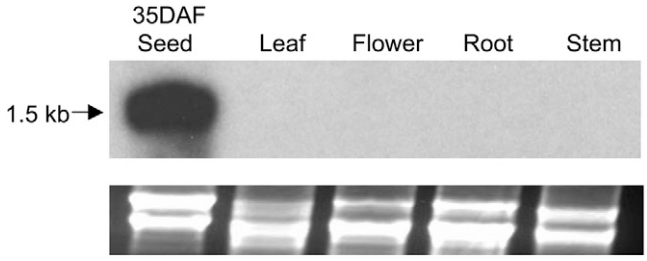

Figure 2. RNA gel blot of *SACPD-C* transcripts in different soybean tissues. Lower panel shows ethidium bromide staining of the gel before blotting to show the relative equivalency of RNA loading. DAF, days after flowering.

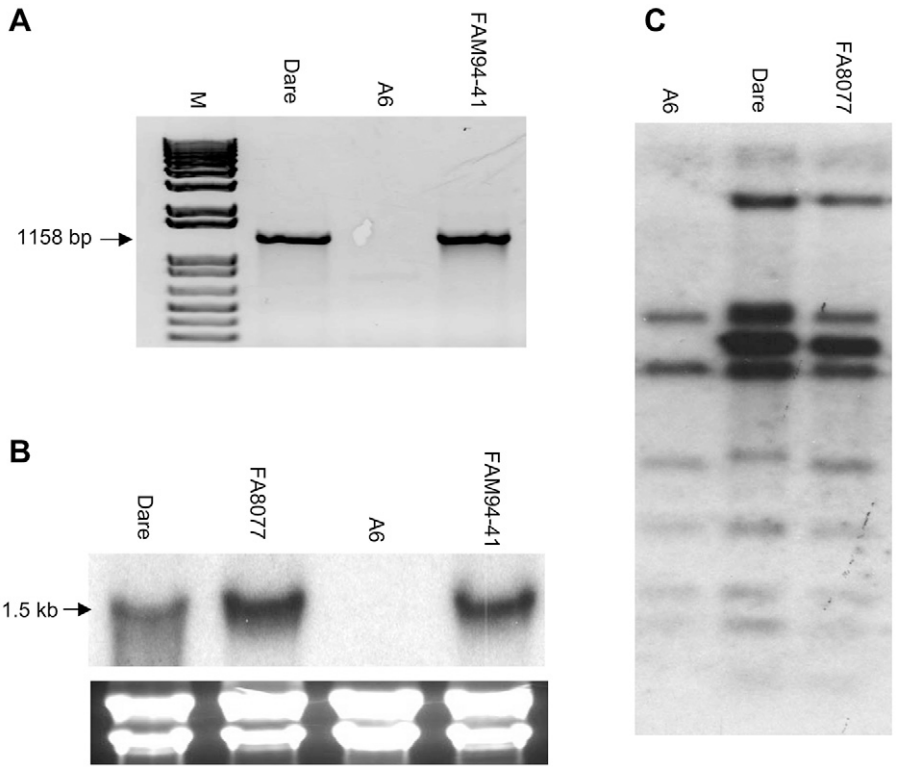

Figure 3. Analysis of *SACPD-C* in A6 and FAM94-41. (A) Reverse transcriptase-polymerase chain reaction amplification of *SACPD-C* using RNAs isolated from developing seeds 35 days after flowering of 'Dare', A6, and FAM94-41. Molecular weight markers are shown in the far left lane. (B) RNA blot analysis of *SACPD-C* transcripts from developing seeds of Dare, FA8077, A6, and FAM94-41. Ethidium bromide–stained gel is shown in lower panel as loading control. (C) DNA gel blot analysis of *Hind* III–digested genomic DNAs from A6, Dare, and FA8077, using radiolabeled *SACPD-C* cDNA as the hybridization probe.

in a nonconserved amino acid substitution in a critical region of the enzyme. As shown in Fig. 4, a G→A polymorphism at position 376 of the cDNA changes an Asp residue to an Asn at position 126 of the predicted amino acid sequence. Alignment of the FAM94-41 protein sequence to the nonredundant protein database of GenBank revealed that this position is highly conserved among plant Δ^9–S-ACP-Des proteins. The acidic residues Asp or Glu are found at the comparable location in every other Δ^9–S-ACP-Des GenBank entry (data not shown). Furthermore, when the SACPD-C polypeptide sequence is superimposed on the three-dimensional structure that has been determined for the castor bean (*Ricinus communis* L.) Δ^9–S-ACP-Des (Lindqvist et al., 1996), Asp^{126} is located within one of the two pairs of antiparallel helices that comprise the catalytic di-iron center of the enzyme, lying immediately adjacent to an invariant Glu residue that serves as an iron-binding ligand (Glu^{105} of the castor enzyme; Glu^{125} of SACPD-C).

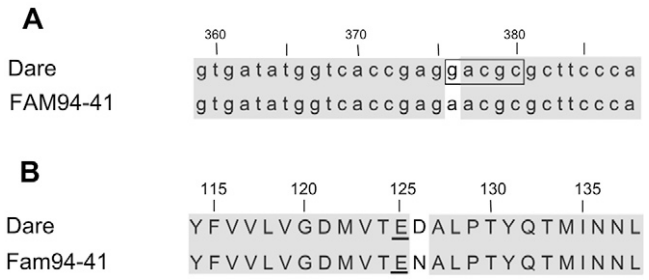

Figure 4. Point mutation in the *SACPD-C* gene of FAM94-41. (A) Sequence comparison of the *SACPD-C* genes from 'Dare' (GenBank no. EF113911) and FAM94-41 (GenBank no. EF113912) in the region surrounding the G→A polymorphism at position 376 (with respect to initiation codon of *SACPD-C* cDNA). A rectangle denotes the recognition motif of the restriction enzyme *Hga* I that is absent in the sequence derived from FAM94-41. (B) Localized amino acid sequence comparison showing the Asp^{126} to Asn^{126} substitution in the predicted SACPD-C enzyme from FAM94-41. Amino acid positions are in reference to the initiator start Met residue. The predicted iron-binding Glu residue at position 125 is underlined.

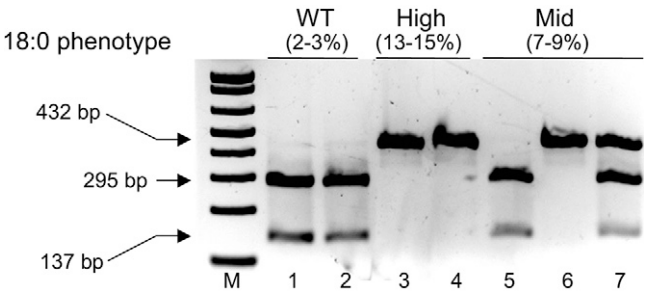

Figure 5. CAPS marker analysis of representative F_2 progeny from a FAM94-41 × N98-4445A–derived population. *SACPD-C*–specific amplification products from select individuals of F_2 families displaying wild-type (WT; lanes 1 and 2), high (lanes 3 and 4), and midpoint (Mid; lanes 5–7) 18:0 phenotypes were digested with *Hga* I and separated on an agarose gel. M, 100-bp molecular weight ladder.

Association Analysis of the FAM94-41 *SACPD-C* Variant

If the Asp^{126} to Asn^{126} polymorphism is indeed responsible for the enhanced 18:0 phenotype of FAM94-41, a perfect correlation should be observed between the elevated stearic acid trait defined by fas_{nc} and the nucleotide polymorphism responsible for the amino acid substitution in *SACPD-C*. The single nucleotide change at position 376 observed between the *SACPD-C* gene from FAM94-41 vs. normal soybean results in the loss of an *Hga* I restriction endonuclease site in the former (Fig. 4A). This polymorphism allowed us to develop a cleavage amplified polymorphic sequence (CAPS) marker (Konieczny and Ausubel, 1993) that can readily distinguish the two alleles. PCR primers were designed that amplified a 432-bp region encompassing the polymorphic restriction site. *Hga* I digestion of the 432-bp *SACPD-C* amplification product from normal soybeans yields fragments of 295 and 137 bp, in contrast to the product derived from FAM94-41 which remains uncut.

Segregation analysis was conducted on an F_2 population of a cross between FAM94-41 and N98-4445A, a mid-oleic acid germplasm line (Burton et al., 2006). Interestingly, introducing the fas_{nc} locus into the mid-oleic background of N98-4445A resulted in higher stearic acid levels (13–15% total fatty acid content) than those observed in its original background (9%; data not shown). Three discrete classes of stearic acid phenotypes were observed when fatty acid analysis was conducted on bulked seed from each F_2 plant: wild-type (2–4% 18:0), high (12–15%), and midpoint (7–9%). Seed from 40 F_2 plants displaying wild-type 18:0 levels, and 47 plants with a high 18:0 content were genotyped using the *SACPD-C* CAPS marker. All lines classified as having a normal 18:0 seed phenotype were homozygous for the wild-type *SACPD-C* allele, and 100% of the F_2 lines displaying an elevated 18:0 phenotype were homozygous for the *SACPD-C* allele derived from FAM94-41. Representative results are shown in Fig. 5. We predicted that the two *SACPD-C* alleles would be segregating in seed bulks from F_2 plants possessing a midpoint 18:0 phenotype. To test this, 10 seed from each of three independent midpoint 18:0 lines were individually genotyped with the *SACPD-C* CAPS marker. As expected, representatives of all three possible marker genotypes were observed in each of the midpoint lines tested. Examples from one line are shown in Fig. 5. The precise cosegregation of the FAM94-41–derived *SACPD-C* marker genotype with the elevated 18:0 trait among the FAM94-41 × N98-4445A F_2 lines tested strongly supports the hypothesis that the aberrant *SACPD-C* allele defines the fas_{nc} locus.

In Vitro Analysis of SACPD-C Enzyme Activity

Given the occurrence of the Asn^{126} substitution in the *SACPD-C* gene in FAM94-41 at a location where an acidic residue normally resides, we speculated that the resultant enzyme (SACPD-C_{FAM}) may be less active than the wild-type enzyme (SACPD-C_{WT}) and thus provide a rational explanation of the elevated 18:0 phenotype observed in this line. To test the relative activities of SACPD-C_{FAM} vs. SACPD-C_{WT}, each enzyme was expressed in *E. coli*, purified, and assayed in the presence of ^{14}C-labeled 18:0-ACP substrate. The recombinant Δ^9–S-ACP-Des enzyme from castor bean was used as a positive control. Surprisingly, both soybean enzymes were far less active than the castor enzyme in catalyzing 18:0-ACP desaturation in vitro (Fig. 6). Equally unexpected, the mutant SACPD-C_{FAM} enzyme consistently displayed ~10-fold higher activity than the wild-type soybean enzyme in these assays.

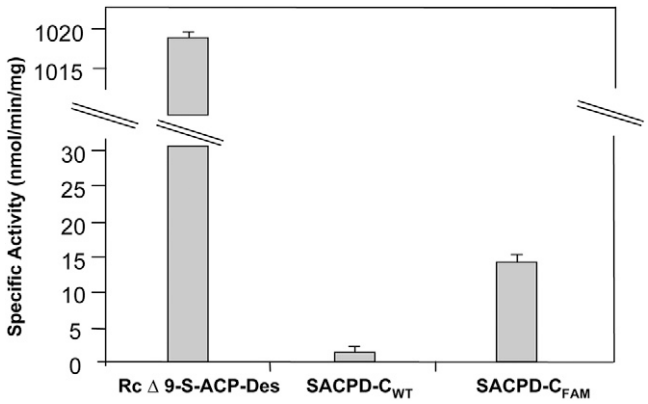

Figure 6. In vitro synthesis of oleic acid using recombinant Δ^9–stearoyl-acyl carrier protein desaturase (Δ^9–S-ACP-Des) proteins. Activities are shown as nmol product min^{-1} mg^{-1} recombinant protein (from preparations >90% pure). Rc, *Ricinus communis* (castor). Error bars represent SE (n = 3 for castor enzyme; n = 5 for SACPD-C_{WT}; n = 4 for SACPD-C_{FAM}).

DISCUSSION

The discovery and characterization of the seed-specific *SACPD-C* gene provides new insights regarding the mechanisms by which the stearic acid content of soybean seeds is controlled. The finding that *SACPD-C* is deleted in the A6 germplasm, and a mutant version of the gene from FAM94-41 faithfully segregates with the high 18:0 phenotype, provides compelling evidence that the encoded enzyme plays a major role in the biosynthesis of oleic acid during soybean seed development. Therefore, the observation that the specific activity of the purified, recombinant wild-type SACPD-C enzyme was >500-fold less than the prototypical castor enzyme (Fig. 6) was unexpected. Several independent replications of the in vitro assay–based experiments invariably yielded the same results, causing us to conclude that SACPD-C is inherently less active than the castor enzyme or FAB2, the predominant Δ^9–S-ACP-Des enzyme of *Arabidopsis* (Kachroo et al., 2007), under the conditions of this assay. Two studies from the laboratory of J. B. Ohlrogge (Michigan State Univ.) have shown that in vitro Δ^9–S-ACP-Des enzyme activity can vary depending on the specific isoforms of ferredoxin (Schultz et al., 2000) or ACP (Suh et al., 1999) that are used as the electron-donating cofactor and the 18:0-ACP substrate, respectively. It is possible that SACPD-C does not interact well with the *Anabaena*-derived ferredoxin and/or spinach ACP that were used in this study. Alternatively, SACPD-C may require a posttranslational modification that is not recapitulated during synthesis in the *E. coli*–based expression system, such as phosphorylation, to attain full activity. The fact that SACPD-C shares <65% amino acid sequence homology with SACPD-A or -B, the *Arabidopsis* FAB2, or the castor enzyme, whereas the latter four enzymes all share >82% identity with each other (Fig. 1), lends plausibility to the speculation that SACPD-C is regulated differently than are typical Δ^9–S-ACP-Des enzymes. Finally, the "mature" recombinant SACPD-C enzyme used in the in vitro assays was engineered based on removal of the predicted chloroplast transit peptide shown in Fig. 1A. If this predicted cleavage site does not truly reflect the in vivo N-terminal maturation of the protein, failure to perfectly reconstitute the correct mature protein could provide another explanation for reduced overall activity.

Equally paradoxical is the observation that SACPD-C_{FAM} showed higher activity than $SACPD_{WT}$ in the in vitro enzyme assays. The proximity of the Asp^{126} to Asn^{126} substitution found in $SACPD_{FAM}$ to the predicted iron-binding Glu^{125} residue, together with the observation that only acidic residues (Glu or Asp) are found at the comparable position in every other soluble Δ^9–S-ACP-Des enzyme sequence deposited in GenBank, caused us to predict that $SACPD_{FAM}$ enzyme activity would be reduced relative to the wild-type. Instead, $SACPD_{FAM}$ proved to be ~10-fold more active than $SACPD_{WT}$ in the in vitro assay (Fig. 6). One plausible explanation for this phenomenon is that although the Asp^{126} to Asn^{126} substitution may result in greater inherent enzyme activity in vitro, it may also lead to greatly reduced stability in vivo. Regardless, the unexpected findings revealed in this study highlight the fact that there remains much to be learned concerning the structure:function relationship of Δ^9–S-ACP-Des enzymes in plants and the mechanisms by which they are regulated.

An interesting pattern is beginning to emerge concerning the organ specificity of genes associated with glycerolipid biosynthesis in soybean. Previously, both seed-specific and non–seed-specific isoforms have been identified for

genes encoding the endoplasmic reticulum–localized FAD2 (18:1 desaturase) and FAD3 (18:2 desaturase) enzymes in soybean (Heppard et al., 1996; Bilyeu et al., 2003; Tang et al., 2005). With our characterization of *SACPD-C*, it is apparent that the soybean genome possesses seed-specific isoforms for all three of the desaturase enzymes expected to be involved in synthesis of storage triacylglycerols. A reasonable explanation for the occurrence of seed-specific isoforms of these genes would be to assist in accommodating the great increase in lipid biosynthesis that occurs as the developing soybean seed is producing storage oil reserves. However, this phenomenon does not appear to be universal for all steps of the glycerolipid pathway that are required for triacylglycerol production, as no seed-specific isoforms were found among the four 16:0-ACP thioesterase genes or the two 3-keto-acyl-ACP synthase II genes that we have previously characterized from soybean (Aghoram et al., 2006; Cardinal et al., 2007).

The *fas*^{a} locus of germplasm A6 can confer a stearic acid seed phenotype of ~30% total fatty acid; however, this locus is also associated with a severe depression in yield (Hartmann et al., 1997). In addition to the possibility that the high 18:0 seed phenotype per se is responsible for the observed yield drag, it is plausible that the deletion event that gave rise to A6 may have also encompassed other genes on the same chromosomal fragment that could impact yield. Although the gains in 18:0 content mediated by fap_{nc} are more modest (13–15% in this study) than those attributed to *fap*^{a}, no yield penalty has been associated with fap_{nc} (Pantalone et al., 2002). Introducing the elevated 18:0 phenotype of the fap_{nc} locus into an elevated oleic acid, low linolenic acid germplasm, such as N98-4445A, represents a promising strategy for the development of soybean lines with greatly enhanced oxidative stability. The fap_{nc}-specific CAPS marker developed in this study could serve as a useful tool in achieving this goal by accelerating the introgression of the elevated 18:0 trait into desired backgrounds through marker-assisted selection.

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